Production, Optimization and characterization of active metabolites from marine yeasts

1. INTRODUCTION

Microorganisms are widely distributed in the marine environment but the exploration of microbes is very limited. The activity and the characteristic feature of marine microbes are different from the terrestrial sources (Carr *et al*., 2003). Their number and distribution depends on organic materials and other environmental factors. Specifically marine yeasts are not only found in sea water, sediments and plants of marine habitats but also in digestive tract of marine animals and sea birds. Marine yeasts are unique in nature than the terrestrial by their unique properties like high osmotic tolerance, higher industrial enzyme productivity (Kutty & Philip *et al*., 2008). Nowadays yeasts are widely applied for the production of commercial products like bio-ethanol, lipids, fatty acids etc (Ewa *et al*., 2017). Lipids are produced by all microorganisms usually in the range of 6 to 8% per dry biomass principally as components for the cell membrane. Microorganisms, including yeasts, bacteria, filamentous fungi and microalgae can synthesize and accumulate more than 20% lipids in their biomass. These microorganisms are called oleaginous organisms (Patel *et al*., 2020). Yeasts are advantageous for lipid production over other sources, as they can accumulate oil up to 80% of their dry weight and their cultures can be easily scaled up. Accumulation of lipids up to 25% -70% of the biomass, also the lipid profile of these yeasts differ between the species (Ageitos *et al*., 2011).

Lipids produced by yeasts have similar fatty acid profiles compared to those of vegetable oils and are therefore considered as an alternative strategy for the production of second-generation fuels, including biodiesel (Robak & Balcerek 2018). The oleaginous yeasts, when compared to other oleaginous organisms can accumulate 40% of lipid and require small area for their cultivation. The main objective of this study is to isolate potential oleaginous yeast that can accumulate more than 40% of lipid with the basal medium. The accumulation of lipid in microorganisms especially in yeast is done through (i) Denovo biosynthesis: Hydrophilic substrates like sugar or carbohydrates is fermented to convert it into lipids; (ii). Exnovo biosynthesis: Hydrophobic substrates like oil and alkanes were converted into lipids (Papanikolaou & Aggelis 2011; Huang *et al*., 2017).

Accumulation of lipid is a complex process that involves many metabolic reactions spanning multiple cellular organelles in eukaryotic cells. The major intermediates in lipid biosynthesis are fatty acids, and are primarily found in lipid bodies for storage.

2. Review of literature

The classical definition of oleaginous yeast is the one that accumulates greater than 20% dry cell weight (dcw) as lipids. *S. cerevisiae* is not known as being oleaginous and typically only accumulates 10–15% of its dcw as lipids (Meng *et al*., 2009; Ratledge 2002; Runguphan and Keasling 2014; Greer *et al*., 2015). Oleaginous yeasts, such as *Rhodosporidium toruloides, Lipomyces starkeyi*, and *Yarrowia lipolytica*, can typically accumulate 25% to greater than 60% lipids (Adrio 2017; Ageitos *et al*., 2011). The extent of lipid accumulation in oleaginous yeast is determined by genetic constitution, as maximum attainable lipid contents can vary enormously among species and even among individual strains (Meng *et al*., 2009).

The accumulated oil is almost invariably in the form of triacylglycerols, the fatty acyl groups of both neutral and polar lipids containing stearic, oleic, linoleic and palmitic acid (Papanikolaou *et al*., 2002). In general yeast contains no unusual and no deleterious fatty acids. The oleaginous yeast can accumulate lipids within a short retention period and also grown well in variety of substrates, low cost material that has nutritional residues (Zhu *et al*., 2008). In order to achieve maximum lipid accumulation in yeast, it has to be grown in a medium with excess of carbon substrate and a limiting amount of nitrogen medium. In that case, the organism exhausts the supply of nitrogen quickly but it continues to assimilate carbon source (Meng *et al*., 2009).

Yeasts are chemoorganotrophic organisms that they can obtain carbon and energy from compounds in fixed, organic linkage. Glucose is the common carbon source most widely utilized by yeasts. *Yarrowia lipolytica* effectively utilizes hydrophobic substrates like alkanes and oils as a sole carbon source (Fickers *et al*., 2005). The maximum specific growth rate of *Rhodotorula minutta* IIP-33 is 0.34 hr^{-1} while using glucose as a sole carbon source (Saxena *et al*., 1998). Similarly lipid production from *Rhodotorula glutinis* was 49.25% while using glucose (100g/L) as a carbon source (Dai *et al*., 2007).

When cells run out of nutrient conditions usually nitrogen, excess substrates continues to be assimilated by the cells and converted into fat for storage. Nitrogen limiting conditions has been used more extensively to create an environment contributing to lipid accumulation (Gill *et al*., 1997).

3. Objective of the study

The objectives of this study are as follows:

1. To isolate and screen lipid accumulating oleaginous yeasts from the extreme marine environment.

2. To optimize the parameters for maximum accumulation of lipid and to characterize the presence of fatty acids through instrumental analysis like Gas Chromatography Mass Spectrometry (GCMS) and Fourier Transform Infrared Spectroscopy (FTIR).

3. To study the toxicity of lipids for aquaculture application.

4. Materials and methods

4.1 Sample Collection and Isolation

Sea water samples were collected at the depth of 2m, 50m, 500m, 1500m, 2000m and 2500m from three different stations in Bay of Bengal using Sagar Nidhi. The following media were used for the isolation of oleaginous organisms. Sabouraud's Chloromphenicol Agar (Loureiro *et al*., 2005), Yeast Extract Peptone and Dextrose Agar (YEPD), Minimal Salt Agar with 2% Dextrose (Ykema *et al*., 1989) were used for the isolation of oleaginous yeasts from the samples. All the media were prepared with 1:1 dilution of filtered sea water and distilled water and autoclaved at 121º for 15-20 minutes. The water samples were serially diluted and plated. The plates were kept at 28±1ºC for 7 days.

4.2 Colony Characterization

The mucoid colonies were selected from the inoculated plates and sub-cultured in glycerol medium (Gientka *et al*., 2017) and glucose agar medium (Ykema *et al*., 1989). The colony morphology of

the selected strains were characterized and the selected cultures were stocked in glycerol for further use.

4.3 Screening of oleaginous yeasts

Preliminary screening of oleaginous yeasts was done using sudan black staining.

4.3.1 Primary screening with Sudan black staining: Sudan black stain 0.3 % was prepared in 70% ethanol and used directly for the staining. Sudan black staining was done according to Kitcha and Cheirsilp (2011). After drying the samples were visualized under microscope.

4.3.2 Secondary Screening by lipid extraction

Lipid extraction was performed following the method of Bligh and Dyer (1959). The isolated yeast cells were inoculated in glucose medium. After 48 hours incubation, the medium was centrifuged at 10000 rpm for 10 minutes to recover the yeast cell pellets. The collected pellets were washed with sterile distilled water. The cell pellets were kept at -80ºC overnight and then subjected to lyophilization. After lyophilization dried cells were weighed around 0.1g and suspended in 5 ml Phosphate Buffered Saline (PBS). The cell suspension was kept for autoclaving at 121ºC for 15 mins. After that cells were allowed to cool and equal volume of Chloroform: Methanol (2:1) was added to it and kept in a shaker at constant rpm for 1 hour. The suspension was centrifuged at 10000 rpm for 5 mins. The upper aqueous phase containing lipid was collected and evaporated in rotary vacuum evaporator. The lipid weight was measured. The percentage of lipid accumulation was calculated according to the formula:

Lipid accumulation (%) = Total lipid (g) / Total biomass (g)

4.4 Optimization of parameters

The important fermentation process parameters like temperature, aeration/mixing, initial pH of culture broth and carbon to nitrogen ratio using the conventional one-factor-at-a time approach (OFAT) was followed. Based on the lipid accumulation screening, only three isolates were selected for shake flask optimization. Minimal nitrogen media (MNM) composition: Glucose- 33 g/L, NH_4Cl- 0.139 g/L, Yeast Extract -1.5 g/L, KH_2PO_4 -3.2 g/L and $MgSO_4.7H_2O$ - 1.0 g/L were used for the optimization studies as a basal medium. As per the conventional OFAT optimization

approach, critical physical and physiological parameters were optimized by varying a single factor at a time. Each subsequent factor was examined after taking into account the previously optimized factor(s). The highest yielding variable in each study was carried forward for the optimization of the next parameter. Lipid weight and biomass were calculated during the optimization studies.

4.4.1 Optimization of carbon source: Seven carbon sources such as glucose, sucrose, lactose, starch, glycerol, palm oil, sunflower oil were used for the optimization studies. The concentration of optimized selected carbon sources was studied from 20 to 60 g/L by replacing the carbon source in the MNM.

4.4.2 Optimization of nitrogen source: Inorganic nitrogen sources such as ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium nitrate and organic nitrogen sources such as urea, yeast extract, peptone and beef extract were used for the optimization studies by replacing the respective nitrogen sources in the MNM. The concentration of optimized organic and inorganic nitrogen sources was studied.

4.4.3 Optimization of pH: The three selected bacterial strains were cultivated in the MNM medium over a pH range of 4.0 to 9.0 for 120hr at fixed rpm of 180.

4.4.4 Optimization of temperature: The cultures under study were subjected to different incubation temperatures (20ºC, 25ºC, 30ºC, 35ºC and 40ºC) for 120hr at fixed rpm of 180.

4.4.5 Optimization of Inoculum percentage: Varying % of inoculums at 0, 5, 10, 15 and 20 grams of 1.00 OD was used to inoculate the production medium flask (50 cm 3 medium/250 cm 3 Erlenmeyer flask). The flasks were incubated at180 rpm and at temperature 30ºC 120 hr and then harvested and contents analyzed.

4.4.6 Growth and lipid accumulation studies: The 5% (v/v) of the pre-inoculum with 1.0 OD (absorbance) was inoculated into the sterile MNM medium and initial absorbance was measured at 600nm. The flasks were incubated in a shaker at a constant rpm of 180 at 25±2°C. Both the temperature and shaking condition were maintained throughout the experiment. The samples were withdrawn aseptically at every 24 h interval and the absorbance was measured at 600nm and gravimetric analysis of lipid estimation was done.

4.5 Characterization of microbial lipids

4.5.1 Fourier Transform Infra Red Spectroscopy: The extracted lipids were dried and subjected to FTIR analysis to analyze the functional groups present in it.

4.5.2 Gas Chromatography Mass Spectrometry: The composition and percentage of fatty acids were identified using GCMS analysis.

4.6 Molecular characterization of the isolates: DNA of the selected isolates was extracted using DNA isolation kit and the concentration was quantified. Further it was amplified using (Internal Transcribed Spacer) ITS rDNA sequencing using universal primers ITS1F and ITS4. The PCR product of 16S rDNA of the isolates was sequenced by Eurofins (Banglore, India) and further comparison was made with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool).

5. RESULTS

5.1 Isolation and colony characterization

A total of more than 300 colonies were identified in 10^3 dilutions. The cells were identified by their different morphology and total colonies were counted. In YEPD agar, more number of mucoid colonies were observed when compared to other media. The mucoid colonies from all three media are noted and those colonies were again sub-cultured in glycerol and glucose media. Overall 67 isolates were selected based on the mucoid characteristics. The viscous sticky growth of selected yeasts was observed in both glucose and glycerol medium.

5.2 Primary screening of oleaginous yeasts

5.2.1 Sudan black staining:

The selected isolates were visualized for lipid accumulation using sudan black staining. Sudan black B stained only the lipid globules and the counter stain saffranin stained the yeast cells. The oleaginous cells show lipid bodies stained blue with the pink cytoplasmic background (lipids

appearing as dark spots within lighter cytoplasm). The non-oleaginous cells were stained completely pink.

5.2.2 Gravimetric determination of total lipid

Totally 67 strains were screened and only 3 strains showed accumulation of lipid above 30% whereas 7 strains showed 20% accumulation. Forty strains showed very less accumulation within 10-20% and 16 strains had accumulation which is less than 10%.

Lipid accumulation in oleaginous yeasts has been initiated mainly the production of commercial lipids. In general, yeasts can accumulate more lipids over bacteria and microalgae. Yeast have high potential as commercial sources of oils (Ratledge, 2004). The present study helped to generate a base line data of the cultivable marine yeast inhabiting different depth zones in Bay of Bengal. The occurrence and distribution of yeasts vary from depth of zones. The diversity of yeast was higher at 500m in Arabian Sea and 1000m in Bay of Bengal (Rosamma Philip *et al*., 2013). Higher nutrients and lower oxygen concentrations occur in the bottom layer as compared to the overlaying water column in deep waters of the Bay of Bengal, suggesting that considerable quantities of organic matter reach the deep sea floor and get oxidized in the bottom layer (Gupta *et al*., 1984). Similarly in the present study also the distribution of yeast was higher at the depth and not at the sea surface.

The yield of lipids from marine yeast can be increased with various optimization methods by simple manipulations in the fermentation conditions (Dias *et al*., 2015; Sitepu *et al*., 2014). Yeasts can utilize organic wastes for lipid accumulation (Zhang *et al*., 2016).

Initially, 67 colonies were selected based on its colony morphology in specified oleaginous yeast growing medium. Sudan black stain is used to identify the morphology of the yeast cells and to confirm the presence of lipids (Thancharoen *et al*., 2017).

It is observed that the distribution of yeast was higher in the station MS3 than MS1 and MS2. The distribution of yeast is very minimal in MS2. Even though the distribution of yeasts was higher in MS3, the presence of oleaginous yeast is very less. The presence of oleaginous yeast in

MS2 station is 7.6% which is 1% higher than the MS3 station. MS1 had the maximum percentage of around 16% than the other two stations. This study is the first report on the distribution of oleaginous yeast in the Bay of Bengal region. Further research is needed on the exploration of oleaginous yeast for commercial application.

5.3 Optimization of parameters

Totally three isolates 53, 56 and 65 were selected based on the percentage of lipid accumulation. In minimal salt medium, different carbon and nitrogen source were modified and optimized to improve the accumulation of lipid. Isolate 53 had higher lipid accumulation around 33% while using glucose (30g/L) and Urea (1.5g/L). Isolate 56 had higher lipid accumulation around 35% while using glycerol (40g/L) and peptone (1g/L). Another selected isolate 65 had higher lipid accumulation around 35% while using glucose (30g/L) and ammonium dihydrogen phosphate (1.5g/L) as carbon source and nitrogen source respectively. The other major factors pH, temperature and inoculums percentage were found to influence the lipid yield. At pH 6, all the three isolates were shown to accumulate maximum lipid. At temperature 25ºC, isolate 53 and 65 were shown higher lipid yield and cell mass. Isolate 56 was found to have maximum lipid accumulation at temperature 30ºC. Both biomass and lipid yield was higher for all the isolates while using 10% of inoculum.

5.4 Growth and lipid accumulation studies:

The growth kinetics of the 3 isolates 53, 56 and 65 was studied at varying time and accumulation of lipid was analyzed gravimetrically. It was found that the isolate 53 has maximum lipid accumulation at 96 hrs and gradually decreased when it reached 120 hrs. The growth and lipid yield of 56 showed that percentage of lipid accumulation was higher during 72 hrs and decline phase was observed after 96 hrs. Lipid yield of 65 showed that percentage of lipid accumulation was higher during 96 hrs and decline phase was observed after 132 hrs.

5.5 Characterization of microbial lipids

5.5.1 Fourier Transform Infra Red Spectroscopy:

FTIR profiles of yeast isolates were represented by the characteristic lipid peaks from the region 3020-2800 cm^{-1}, 1800-1700 cm^{-1}, 1500-1300 cm^{-1}, 1100-1200 cm^{-1} and 800-700 cm^{-1} (Shapaval *et al*., 2019). FTIR profiles of the isolates 53 and 56 contains stretching at 3020 cm^{-1} representing=C-H stretch in fatty acids; isolate 65 at 2955 cm^{-1} and 56 at 1375 cm^{-1} representing acyl chain of fatty acids; 2930 cm^{-1} and 2850 cm^{-1} for isolate 53 and 56 representing CH_2 of acyl chains in fatty acids, and 1750 cm^{-1} for isolate 65 representing C=O stretching in ethyl esters and indicates the presence of lipids.

5.5.2 Gas Chromatography Mass Spectrometry:

The fatty acid profiles of the isolates were studied using Fatty acid Methyl Ester analysis. The major peaks in isolates 53 and 56 were found to be similar with unsaturated fatty acids and the percentage of unsaturated fatty acid presence was 40 and 25% respectively. In case of isolate 65 the saturated fatty acid accumulation was dominant and it exceeded more than the presence of unsaturated fatty acids.

5.6 Molecular characterization of the isolates:

The amplified product was nearly 500 to 700 bp of size. The sequencing results were trimmed and edited into Fasta format using Bioedit tool and strain names were identified using NCBI BLAST search tool. The isolate 53 showed 92% similarity with *Meyerozyma guillermondi*. The other isolates 56 and 65 showed 94% similarity with *Meyerozyma guillermondi* and *Candida* sp respectively.

Conclusion:

In conclusion, the oleaginous yeast *Meyerozyma* sp. could assimilate glucose and glycerol to accumulate intracellularly a considerable amount of lipid content in the cell biomass. The crude lipid contents of the biomass could be increased with the addition of carbon sources and with the adjustment of the C/N ratio of the culture media. The abundant fatty acid contents the lipids will ensure the mass production in the field of aquaculture. The results are discussed in the thesis.

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PAPER PUBLICATIONS

1. **Jasmin Vigila J** , Arunagirinathan N , Selvam R , NagamNaren Rao , Ditto Sharmin D , Revathi K, Sundaravalli .Urinary Tract Infections during Pregnancy in IJIRSET Vol. 8, Issue 7, July 2019.
2. **Jasmin Vigila J,** Revathi K, Shanmuga Swathantham S. Antibiotic Resistance Pathogen causing Infection in Human. Vol.21 No.8(1), 2020. Journal of Natural Remedies. **Scopus**
3. **Jasmin Vigila J,** Dr. K. Revathi, David Ditto Sharmin, Jaideep Mahendra, D. Anandhi, Shanthi Sharmin, M. Arun. Antibacterial efficacy of Oregano Oil (ORIGANUM VULGARE) on the type of bacteria and its count in Dental Aerosols. Vol. 25, Issue 5, Research Gate. **Scopus**

Awards and Honours :

Received **Best oral presentation Award** in the year 2019 for the paper **J Jasmin Vigila**, K Revathi, N Arunagirinathan, R Ponnu Lakshmi 2019 Microbial Analysis of pond water in Kancheepuram District, Tamil Nadu.

Best outstanding Research Scholar National Award 2020 by Kamarajar Institute of Education and Research (KIER**).** Theni, Tamil Nadu, India.